In Vitro Selection of the RNA Aptamer against the Sialyl Lewis X and Its Inhibition of the Cell Adhesion

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Sialyl Lewis X (sLeX) is a tetra-saccharide glycoconjugate of membrane proteins. It acts as a ligand for the selectin proteins during cell adhesion of inflammatory process. Aberrant overexpression of sLeX is also a characteristic of various cancer cells, especially for highly malignant ones. In this paper, the sLeX-specific RNA aptamer was selected using a random RNA library and its affinity and specificity were measured by Surface Plasmon Resonance technique. Affinity of the selected RNA was increased about 1000-fold as compared with the original RNA pool. RNA aptamer bound more specifically to its cognate sugar than to any other similar sugars. Inhibition of the cell adhesion was also shown by in vitro static assay of sLeX-expressing HL60 cells to the E- and P-selectins. It suggests that the high affinity carbohydrate specific RNA aptamer could be used as an alternative to the antibody. © 2001 Academic Press

Key Words: RNA combinatorial library; in vitro selection; RNA aptamer; Sialyl Lewis X; E-selectin; P-selectin; cell adhesion.

Carbohydrates are important mediators in the specific recognition and adhesion between cells (1). Sialyl Lewis X (sLeX) is one of the glycans on the cell surface and is also known as a ligand for the selectin proteins (L-, E-, and P-selectins) (2–4). Selectin-sLeX interaction seems to play a critical role in the initial stage of the inflammation and the cancer cell metastasis (5–9). Considering roles of sLeX in these processes, a blocker for sLeX-selectin interaction would be useful as an anti-inflammatory or as an anti-metastasis drug (10–13). Mimicry of sLeX has been developed by several groups of researcher as potential therapeutics (14). However, chemical synthesis of sLeX mimetics has limited its use as a practical method not only by the low yield of synthesis but also by the low binding affinity and poor specificity of small molecules. Various antibodies also have been developed as molecules recognizing sLeX and blocking the sLeX-selectin interaction (15, 16). However, broad specificity and moderate binding affinities of antibodies might hinder their uses as specific and tight binding molecules (17, 18).

Combinatorial RNA library and affinity selection might have been an alternative method for generating RNA molecules with high affinity and specificity (named as “aptamer”; 19, 20). Previous attempt to select L-selectin binding aptamer resulted in specific L-selectin binding antagonists, which exhibits inhibition of the L-selectin mediated cell adhesion (21, 22). It suggested that the SELEX (Systematic Evolution of Ligand by Exponential enrichment) procedure was extremely useful method for developing specific aptamers for the cell surface proteins. However, no one ever reported a selection of aptamers for the ligand of the selectin, sLeX carbohydrate. Considering high concentration of sLeX glycoconjugates on leukocytes and cancer cells, the cell surface carbohydrate could have been a more effective target for prohibiting sLeX-selectin mediated cell adhesion during inflammation and metastasis.

In this report, we employed the RNA combinatorial library and in vitro selection method to develop the sLeX binding RNA aptamers. Affinities of selected RNA aptamer were subnanomolar to nanomolar range with high specificity to the sLeX. The RNA aptamer inhibited the adhesion of sLeX rich HL60 cells to E- and P-selectin, suggesting that it could be used as a potential cell adhesion blocker. Our results in this report suggest that RNA aptamer against a carbohydrate could be used as a useful lectin, especially as a plausible substitute for antibody against the carbohydrates.

MATERIALS AND METHODS

Cells and other reagents. HL60 cells were maintained at 37°C in RPMI1640 (GIBCO BRL) supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin. Normal mouse IgM was obtained from Sigma and the monoclonal anti-sLeX antibody (KM93)
was purchased from Seikagaku Co. SLel and SLel-BSA were from Oxford Glycoscience, BSA and recombinant soluble E- and P-selectins were from Calbiochem. All other chemicals were purchased from Sigma or Aldrich and used without further purification.

Preparation of affinity matrix. Two hundred milligrams of cyano- gen bromide (CNBr) activated agarose beads was washed with 2.5 ml of 10 mM diethylpyrocarbonate-treated NaOAc (pH 4.5) to remove lactose stabilizer. Activated beads were washed 5 times with 2.5 ml of 100 mM Hepes buffer (pH 8.0). To prepare SLel-conjugated column, the beads were allowed to mix with 2.5 ml of 10 µM SLel solution in Hepes buffer for 2 h at room temperature with stirring. Control bead was also prepared by the same method, except omitting SLel in the binding buffer. To block unbound activated groups, 0.25 ml of 1 M n-butyramine was added to a resulting suspension and stirred for 2 h at room temperature. Beads were then washed again with 2.5 ml of Hepes buffer and stored at 4°C. To be used as affinity matrices, prepared beads were washed and pre-equilibrated with RNA binding buffer [150 mM NaCl, 20 mM Hepes (pH 7.4), 1 mM CaCl₂, 1 mM MgCl₂] just before use.

Preparation of the RNA library. Random DNA library was synthesized by the Midland Company (Midland Certified Reagent Co.). Random region was composed of 70 nucleotides flanked by 5’ and 3’ primer regions. At the primer regions, T7 promoter binding sequences and a few restriction sites were included for the in vitro transcription and the cloning process, respectively. Therefore, 5’- and 3’-primers for PCR and in vitro transcription was 5’-CGAATACTGACTATAGGAGGAGCTCGTTACCAATTTCCG-3’ and 5’-AAGGATCCATCTGATGTGAGCCAGCTG-3’, respectively. Ten tubes of polymerase chain reactions (PCRs) were performed with 57.5 µM of 110-mer DNA random library, 200 µM each of 5’- and 3’-primers, 1.6 mM MgCl₂, 2.5 mM each of dNTPs, 1 U/µl of Taq DNA polymerase. DNA library (3.5 × 10^10 molecules) was converted to the RNA library by in vitro transcription reaction in 50 mM Tris (pH 7.5), 7.5 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 100 mM DTT, 0.5 mM each of rNTPs, 1 U/µl of RNase inhibitor, 50 U of T7 RNA polymerase for 2 h at 37°C. To make the original RNA pool, PCR was amplified by primers containing recognition sites for AMV reverse transcriptase (Promega) and amplified by 20 cycles of PCR. After confirming the PCR products by 2% agarose gel, DNA was transcribed to RNA by in vitro transcription as described above. The RNA band of right size was purified by eluting from 6% PAGE/7 M urea gel. Correct size RNA was isolated from the gel and the radioactivity was measured by the scintillation counter. About 10^-10^ cpm of labeled RNA was incubated either with agarose bead or with SLel-conjugated agarose bead in RNA binding buffer for 30 min in room temperature as above. Unbound RNA was removed by washing the beads 3–4 times; bound RNA was eluted from the bead by phenol extraction, loaded to 6% PAGE/7 M urea gel. The gel was dried and the bound RNA was directly visualized by autoradiography.

Biosensor assay. Blaocore 2000 was used for the surface plasmon resonance experiments. To attach SLel ligand to the CMS sensor chip, surface of the chip was prequillibrated with Hepes and activated with 0.05 M of N-hydroxysuccinimide (NHS) and 0.2 M of N-ethyl-N’-(dimethylaminopropyl)carbodiimide (EDC) by modifying carboxy methyl group. After activation, SLel-BSA (12.6 µl of SLel/mol BSA) solution was injected to one of the flowcell and BSA to the other. After immobilization of the ligand, the chip surface was deactivated with 1 M ethanolamine hydrochloride, pH 8.5. After stabilizing the base line, various concentrations (500, 250, 125, 62, and 31 nM) of the original RNA pool, SELEX cycle 17 RNA and cloned RNA were injected to measure K_D values of these RNA. After each analyte injection, 10 mM EGTA was used to regenerate the ligand surface.

To test the specificity of selected RNA to SLel, RNA was immobilized to sensor chip, and various carbohydrates were analyzed for their binding to RNA. First, 5’-ends of original RNA and the selected clone 5 RNA were biotinylated with oligonucleotide biotin labeling kit (Amersham Life Science). Biotinylated RNA samples were then immobilized to two different flowcellS of the streptavidin (SA) sensor chip. SLel-specific KM-93 monoclonal antibody and control normal mouse IgM were also immobilized to the other flowcells for comparison. Many different types of carbohydrates, including sialyl Lewis X, Lewis X, sialyl Lewis A, and Lewis A were injected as analytes. At least five different concentrations of each carbohydrate were injected to measure binding constants.

Cell adhesion assay. Recombinant soluble E-selectin or P-selectin (Calbiochem) was added to the microtiter plate (250 ng/well) in 0.05 M NaHCO₃ at pH 9.2 (10 µg/ml) and incubated overnight at 4°C. TNF-α activated (10 ng/ml for 20 h) HL60 human promyelocytic leukemia cells were harvested and suspended to a density of 1 × 10⁵ cells/ml in RNA binding buffer [150 mM NaCl, 10 mM Hepes (pH 7.4), 1 mM CaCl₂, 1 mM MgCl₂]. Fifty microliters of cell suspension was pipetted into the wells and incubated for 15–30 min at room temperature. Cells were washed with washing solution (DEPC-treated 1× PBS, 1 mM CaCl₂, 1 mM MgCl₂) until no floating cells were detected. BSA was also coated to the plate to measure nonspecific bindings. To measure the inhibition of cell adhesion by RNA molecules, cells were preincubated with various amounts of the original RNA, the clone 5 RNA and the yeast tRNA for 30 min at room temperature and the numbers of adhered cells were measured as described above. Adhered cells were counted and averaged from 10 different microscopic fields, excluding the highest and the lowest numbers from each set of data. For each data, duplicate reactions were performed.

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RESULTS

Selection of sLeX-Specific RNA

In order to select the sLeX-specific RNA, the affinity matrix for the sLeX was prepared by attaching sLeX to the agarose beads using hydroxyl functionality in the sLeX (Fig. 1A; 23). The 110-mer DNA library was designed to contain random nucleotides in the central 70 positions flanked by the defined sequences at each end. T7 promoter was placed at the 5' of the random sequences for in vitro transcription; various restriction sites were included at each end for the PCR amplification and for the cloning of selected sequences (Fig. 1B). To remove non-specific RNA binding to the agarose, RNA molecules were first loaded to the agarose precolumn and the unbound fraction was loaded to the sLeX-immobilized column. After washing the column with excess binding buffer, sLeX bound RNA was affinity eluted with the sLeX solution in the early cycles or with high salt and EDTA in the later cycles. The eluted RNA was then reverse transcribed and amplified by PCR for the next round of the selection. After enriching RNA by 17 cycles of selection, RNA molecules were cloned into pBluescriptII-KS using SacI and BamHI sites in 5' and 3'-ends, respectively. Specific sequences were enriched as shown in Fig. 1C.

Selected RNA Binds to sLeX with High Affinity

We evaluated the binding affinity of the selected RNA by two methods. First, RNA was radiolabeled,
allowed to bind to the sLe\(^\text{X}\) conjugated agarose bead and its binding was visualized by gel electrophoresis and autoradiography (Fig. 2). RNA from 17 rounds of selection (SE) binds strongly to the sLe\(^\text{X}\) (sLe\(^\text{X}\) lane), while it hardly binds to the agarose bead (B lane), suggesting that the RNA aptamer specifically binds to sLe\(^\text{X}\) but not to agarose bead itself. In contrast, almost no original RNA pool (Ori) binds either to the sLe\(^\text{X}\) or to the agarose bead.

Secondly, the surface plasmon resonance (SPR) measurement was employed to accurately assess the binding affinities of the selected RNA pool and the cloned RNA aptamers (24). After immobilizing the sLe\(^\text{X}\)-BSA and the control BSA into each flowcell of the sensor-chip, RNA solution was injected to the chip. Selected RNA bound more strongly to sLe\(^\text{X}\)-BSA over to BSA, suggesting that the selected RNA specifically bound to sLe\(^\text{X}\)-BSA, but not to BSA protein. The binding parameters of many different RNA molecules were determined by injecting six different concentrations of RNA (Table 1). Binding affinity of the selected RNA was increased 1000-fold, up to subnanomolar scale as compared to the original pool of RNA. Interestingly, all of the selected clones showed similar or even better binding affinities to sLe\(^\text{X}\) than the commercially available anti-sLe\(^\text{X}\) antibody KM-93 did. It suggests that the in vitro RNA selection against the carbohydrates might generate higher affinity aptamers, which could substitute for lower affinity antibodies for non-antigenic small molecules.

**Specificity of the Selected RNA**

Since the clone 5 RNA was found to have the maximum binding affinity, we utilized it as a model aptamer for testing the specificity. Clone 5 RNA was immobilized to the sensor chip and various carbohydrates were injected for the SPR measurement. To immobilize the RNA molecules without disturbing native structure, the 5′-end of the RNA was biotinylated and attached to the streptavidin-coated sensorchip.

<p>| TABLE 1 |
| <strong>Binding Affinity of the Selected RNA Aptamers to sLe(^\text{X})</strong> |</p>
<table>
<thead>
<tr>
<th>RNA</th>
<th>(k_a \ (\text{I/M sec}))</th>
<th>(k_d \ (\text{I/sec}))</th>
<th>(K_a \ (\text{I/M}))</th>
<th>(K_d \ (\text{M}))</th>
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<tr>
<td>Original pool</td>
<td>(2.6 \times 10^4)</td>
<td>(3.3 \times 10^{-3})</td>
<td>(7.6 \times 10^{-6})</td>
<td>(1.3 \times 10^{-7})</td>
</tr>
<tr>
<td>Selected pool</td>
<td>(2.4 \times 10^5)</td>
<td>(1.4 \times 10^{-3})</td>
<td>(1.7 \times 10^{-5})</td>
<td>(5.8 \times 10^{-10})</td>
</tr>
<tr>
<td>Clone 5</td>
<td>(1.3 \times 10^5)</td>
<td>(1.1 \times 10^{-5})</td>
<td>(1.1 \times 10^{-6})</td>
<td>(8.5 \times 10^{-10})</td>
</tr>
<tr>
<td>Clone 2</td>
<td>(9.8 \times 10^3)</td>
<td>(7.3 \times 10^{-5})</td>
<td>(1.2 \times 10^{-7})</td>
<td>(8.0 \times 10^{-10})</td>
</tr>
<tr>
<td>Clone 15</td>
<td>(3.5 \times 10^3)</td>
<td>(8.1 \times 10^{-6})</td>
<td>(4.3 \times 10^{-8})</td>
<td>(2.3 \times 10^{-9})</td>
</tr>
<tr>
<td>Clone 18</td>
<td>(5.1 \times 10^3)</td>
<td>(2.0 \times 10^{-3})</td>
<td>(2.5 \times 10^{-6})</td>
<td>(3.9 \times 10^{-9})</td>
</tr>
<tr>
<td>Clone 4</td>
<td>(4.1 \times 10^3)</td>
<td>(3.1 \times 10^{-5})</td>
<td>(1.3 \times 10^{-7})</td>
<td>(7.4 \times 10^{-9})</td>
</tr>
<tr>
<td>Clone 9</td>
<td>(3.5 \times 10^3)</td>
<td>(3.1 \times 10^{-5})</td>
<td>(9.5 \times 10^{-7})</td>
<td>(1.0 \times 10^{-8})</td>
</tr>
<tr>
<td>Anti-sLe(^\text{X}) Ab</td>
<td>(2.5 \times 10^3)</td>
<td>(1.7 \times 10^{-5})</td>
<td>(1.5 \times 10^{-6})</td>
<td>(6.7 \times 10^{-9})</td>
</tr>
</tbody>
</table>

Note. Binding parameters of the sLe\(^\text{X}\) to the various RNA molecules and the antibody was measured by the SPR technique. sLe\(^\text{X}\)-BSA and BSA were immobilized to different flowcells of biosensor CMS chip and at least five different concentrations of various RNA and antibody were injected to determine kinetic parameters. After subtracting background binding to BSA, binding parameters for sLe\(^\text{X}\)-BSA were evaluated using BIA evaluation program. Only the data that meet the evaluation criteria are presented here.

The \(K_d\) value of the RNA aptamer for the sLe\(^\text{X}\)-BSA was much higher than for that of BSA (Table 2), confirming the previous result. Binding parameters of the clone 5 RNA aptamer to various carbohydrates were also presented in Table 2. The RNA aptamer tightly bound to the cognate molecule, sLe\(^\text{X}\), followed by Le\(^\text{A}\), sLe\(^\text{A}\) and Le\(^\text{A}\). Even though the binding affinity to the sLe\(^\text{X}\) is only 5–10 times stronger than similar Lewis group sugars, it is 100 times higher than that of the dissimilar sugar, such as the lactose. Above binding data suggested that the selected RNA aptamer could discriminate the minor differences in carbohydrates, with the highest affinity to its cognate sugar, sLe\(^\text{X}\).

<p>| TABLE 2 |
| <strong>Specificity of the RNA Aptamer</strong> |</p>
<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>(k_a \ (\text{I/M sec}))</th>
<th>(k_d \ (\text{I/sec}))</th>
<th>(K_a \ (\text{I/M}))</th>
<th>(K_d \ (\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>sLe(^\text{X})-BSA</td>
<td>(6.4 \times 10^7)</td>
<td>(3.7 \times 10^{-3})</td>
<td>(1.7 \times 10^{-5})</td>
<td>(5.7 \times 10^{-11})</td>
</tr>
<tr>
<td>BSA</td>
<td>(2.2 \times 10^6)</td>
<td>(2.3 \times 10^{-3})</td>
<td>(9.9 \times 10^{-6})</td>
<td>(1.0 \times 10^{-7})</td>
</tr>
<tr>
<td>sLe(^\text{X})</td>
<td>(1.7 \times 10^5)</td>
<td>(5.5 \times 10^{-4})</td>
<td>(3.0 \times 10^{-5})</td>
<td>(3.3 \times 10^{-9})</td>
</tr>
<tr>
<td>sLe(^\text{A})</td>
<td>(1.2 \times 10^5)</td>
<td>(1.9 \times 10^{-5})</td>
<td>(5.9 \times 10^{-6})</td>
<td>(1.7 \times 10^{-8})</td>
</tr>
<tr>
<td>Le(^\text{X})</td>
<td>(6.7 \times 10^4)</td>
<td>(1.6 \times 10^{-5})</td>
<td>(4.1 \times 10^{-6})</td>
<td>(2.4 \times 10^{-8})</td>
</tr>
<tr>
<td>Le(^\text{A})</td>
<td>(7.3 \times 10^4)</td>
<td>(1.0 \times 10^{-5})</td>
<td>(7.2 \times 10^{-6})</td>
<td>(1.4 \times 10^{-8})</td>
</tr>
<tr>
<td>Lactose</td>
<td>(6.3 \times 10^3)</td>
<td>(1.8 \times 10^{-4})</td>
<td>(3.4 \times 10^{-6})</td>
<td>(2.9 \times 10^{-7})</td>
</tr>
</tbody>
</table>

Note. Binding parameters of the done 5 RNA aptamer to sLe\(^\text{X}\)-related various sugars as measured by the SPR technique. Clone 5 RNA and Original RNA library were biotinylated and immobilized to the different flowcells of the streptavidin-coated biosensor chip. Various concentrations of the carbohydrates were injected and the binding parameters were determined. Specific binding to the clone 5 RNA aptamer was calculated for each sugar, after subtracting background binding to the original RNA library. Specificity of RNA aptamer was also confirmed by injecting sLe\(^\text{X}\)-BSA or BSA to the immobilized clone 5 RNA aptamer.
Inhibition of the HL60 Cell Binding to E- and P-Selectins

Since the selected RNA aptamer binds to sLeX with high affinity and specificity, it is presumed to act as a blocking agent for the sLeX-mediated cell adhesion. We therefore tested whether the clone 5 RNA aptamer could inhibit the cell adhesion mediated by the sLeX and the selectin proteins. Static microtiter cell adhesion assay was performed by immobilizing recombinant E- or P-selectins on the plate, followed by incubating with sLeX expressing cells on the protein coated wells. Promyelocytic leukemia cell line HL60 was used in this study, because the surface of the cell was rich in sLeX, especially after TNF-α treatment (25). The number of HL60 cells that adhered to the E-selectin coated wells was much higher than that of control BSA-coated wells, as expected (Fig. 3A). To test whether the RNA aptamer could specifically inhibit the HL60 adhesion to the E-selectin by blocking the cell surface sLeX, various amounts of the clone 5 RNA aptamer were incubated with the HL60 cells and allowed to bind to the cell surface sLeX. As the amount of the RNA aptamer was increased, the adhered cells to the E-selectin coated plates were significantly reduced. Such inhibitory effect of the clone 5 RNA aptamer is not resulted from nonspecific binding of the RNA, because the same amount of tRNA did not reduce the cell adhesion. Control experiments were also performed to confirm the specific inhibition of the sLeX mediated cell adhesion.

Similar results were obtained when HL60 cells were incubated with P-selectin coated plates (Fig. 3B). Clone 5 RNA aptamer inhibited cell adhesion, whereas tRNA did not reduce or even increased the adhesion of HL60 to P-selectin. These results suggest that most of the interaction between E- and P-selectin and the sLeX on the HL60 cells are specific and could be inhibited by the sLeX binding RNA aptamer. More studies needs to be done to demonstrate the inhibitory effect of the RNA aptamer.

DISCUSSION

In this study, we utilized the in vitro RNA selection method to develop novel RNA aptamers to the sLeX sugar of the cell surface glycoproteins. Selection from a large pool of the original random RNA (3.5 × 10^14 molecules) resulted in the enrichment of the specific RNA aptamers. Binding affinity was increased about 1,000-fold after 17 rounds of iterative selections. Selected RNA aptamer was found to have higher binding affinity to its cognate sugar, sLeX and similar Lewis group sugars, but much lower affinity to dissimilar ones.

Large combinatorial pool of RNA could adopt various conformations that could bind to the target molecules with high affinity and specificity (26–28). In vitro selection of the RNA library has been an extremely useful method for isolating high affinity RNA aptamers against various proteins and small molecules (29–32). Sugar molecules could specifically bind to RNA, as demonstrated by the selection of aminoglycoside binding RNA aptamers (33–35). Selection of the RNA aptamers against aminoglycosides could have been relatively easily achieved due to the strong charge-charge interactions between protonated amino functionalities in aminoglycosides and negatively charged phosphate backbones in the RNA molecules. However, the interactions of RNA molecules with the carbohydrates in the cell surface were rarely studied, in spite of their potency as therapeutic targets.

Selection of the RNA aptamer that binds to other neutral sugar, such as the cellubiose unit of cellulose, has been reported (36). It suggests that carbohydrate-binding RNA aptamer could be selected, even though it has relatively low affinity (around 10^-5 to 10^-7 M) and broad specificity. In this study, we have shown that the Kd of sLeX binding RNA aptamer is around 10^-9 to 10^-11 M and it has high specificity for the sLeX related sugars. To our knowledge, this is the first report on the selection of RNA aptamer that has nanomolar affinity with specificity against carbohydrate antigen in cell surface glycoconjugates.

Selection of RNA and/or DNA aptamer against L-selectin has been previously shown to have an an-
agonistic effect for the binding (21, 22). Even though the flow-cell rolling assay needs to be done to directly demonstrate the inhibition of cell adhesion, the RNA aptamer against the sLe\(^\alpha\) sugar was likely act as an inhibitor for the cell adhesion, as shown in this study. Specific inhibitors for cell adhesion mediated by selectin and sLe\(^\alpha\) is hoped to be an extremely useful therapeutic agents for chronic inflammation and cancer cell metastasis. High affinity sLe\(^\alpha\)-specific RNA aptamer that we presented in this paper might shed light on the development of a lead molecule for the cell adhesion blocking anti-inflammatory therapy.

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